Engineering the extracellular matrix to model diseases and orchestrate regeneration

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract:

The extracellular matrix is not only a scaffold to which cells attach, but it is also a matrix that communicates cell signals. Because of the interplay between cells and the extracellular matrix, changes in the extracellular matrix can steer cell fate. This opens up the opportunity to design and engineer the extracellular matrix to communicate changes to the cells. Thus, this thesis has focused on understanding which parameters and signals influence cells, but also on how to utilise this knowledge to engineer a completely defined extracellular matrix. The extracellular matrix can be modulated in several ways, such as cell attachment, degradation properties, porosity, stiffness as well as being easily functionalised with molecules of interest using click chemistry.

Two of the papers in this thesis focus on the development of new tools for glycosaminoglycan research to get a better understanding of how this can be modulated to steer cell signalling. Glycosaminoglycans bind growth factors, which can then either act as a co-receptor to increase the potency of the growth factor or to protect the growth factors from being broken down or inactivated. The tools that we have developed open the possibility to better study the production of glycosaminoglycans from different types of cells and better understand what changes occur in glycosaminoglycan synthesis during disease.

The second two papers in this thesis focus on understanding the extracellular matrix. Article number one focuses on the effect of different extracellular matrices and stretch on cells and their secretome. Article number two, which has been the focus of this thesis, utilises the new findings in the other articles about glycosaminoglycans and the extracellular matrix to create a synthetic and defined extracellular matrix. This extracellular matrix is modified with glycosaminoglycans to have a slow release of growth factors to instruct cells to differentiate both *in vitro* and *in vivo*.

Keywords: Glycosaminoglycan, regeneration, extracellular matrix, scaffold, engineering, lung

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To those who have helped me along, as this has not been a journey alone

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Abbreviations

B4GALT7	Beta-1,4-galactosyltransferase 7
BMP	Bone morphogenetic protein
CRISPR	Clustered regularly interspaced short palindromic repeats
COVID -19	Coronavirus disease 2019
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELR	Elastin like-recombinamer
ELISA	Enzyme-linked immunosorbent assay
FGF2	Fibroblast growth factor 2
GAGs	Glycosaminoglycans
HEK293	Human embryonic kidney 293 cells
HGF	Hepatocyte growth factor
HPLC	High-performance liquid chromatography
PAP	3′-phosphoadenosine 5′-phosphate
PAPS	3′-phosphoadenosine-5′-phosphosulfate
PDGF	Platelet-derived growth factor
RNA	Ribonucleic acid
gRNA	Guide RNA
SPR	Surface plasmon resonance
UMAP	Uniform manifold approximation and projection
VEGF	Vascular endothelial growth factor

Aims

The overall aim of this thesis has been to create tools and to understand the properties needed to engineer a cell-instructive biomaterial.

Paper I

The aim of paper I was to understand the effect and size of the following parameters: patient heterogeneity, cyclic stretch and 3D morphology of the extracellular matrix.

Paper II

The goal of paper II was to create a reproducible biomaterial with a modular pore structure and with the ability to functionalise it with defined glycosaminoglycans.

Paper III and IV

For papers III and IV, the aim was to develop new molecular tools to better understand the role of glycosaminoglycans in disease and regeneration.

Introduction

"If we knew what it was we were doing, it would not be called research, would it?"

- Albert Einstein.

Engineering is often defined as the use of scientific principles to design and build. Instead of using bricks and mortar to build houses, this thesis is about using the extracellular matrix to build a scaffold (biomaterial) that can steer cells. This thesis focuses on the building blocks of the extracellular matrix and the effect of each change. In this study, we designed and built, engineered, the extracellular matrix using a bottom-up approach to create a scaffold with properties to either mimic and study disease or to signal and orchestrate regeneration. Starting with the last two papers, the focus was on the glycosaminoglycans in the body, and the development of new techniques to study them. Glycosaminoglycans are the traffic lights of the extracellular matrix, they bind growth factors that communicate to cells where to go and do spatially, thus a crucial part in trying to instruct cells. The first paper explores several factors which can influence cell behaviour: stretching of the extracellular matrix, the spatial composition of the extracellular matrix, and patient differences. All these factors were explored to study the size effect of each condition. Thereby, we learned that the spatial composition and the type of scaffold were the most influential. The main focus has been on the second paper, where we combined all the findings, using the glycosaminoglycans to steer the cells, but also to engineer a scaffold that had spatial composition mimicking the distal part of a healthy lung. Thus, we were able to combine all the factors explored previously, but still retain the possibility to change and modify the scaffold as we continue to learn and engineer our way into the future.

How the extracellular matrix instructs the body

"Most practising scientists focus on 'bite-sized' problems that are timely and tractable. The occupational risk is then to lose sight of the big picture."

- Martin Rees

In science, we like to focus on a single question at a time, like how does the stretching of the lung affect cells[1], how high is the patient variability[2] or how do different culturing conditions change the cells' behaviour [3]? The extracellular matrix (ECM) plays a fundamental role in transmitting signalling in terms of its composition, structural properties, conformation and cell signalling factors binding and release. In paper I, we explored the impact of how the conformation of the ECM in combination with cyclic stretch affected the cellular response. We cultured patient-derived cells in six different culture conditions, changing the environment as well as combining it with cyclic stretch. Analysing the secretome and the effect of each condition on immune cells, revealed that the environment had the largest effect on the cells and cyclic stretch had the lowest effect.

Extracellular matrix

For a long time, the extracellular matrix was seen as an inert scaffold holding the cells in place. However, increasing evidence is showing that there is an intricate interplay between cells and the ECM[4–6]. The ECM consists of several different proteins, sugars (glycosaminoglycans), and other molecules. Various collagens comprise nearly one-third of the total proteins in the body. Twenty-nine different collagens are known in humans, where collagen I is the most common[7]. Since collagens consist of three-helix chains, which form a super helix, these proteins have impressive mechanical strengths and are, therefore, a crucial part of the ECM[8]. While collagen gives the extracellular matrix its stiffness and rigidity, elastin, as the name suggests, gives the extracellular matrix its viscoelastic properties[8]. This property makes it great at absorbing energy as well as stretching and is, therefore, found in the cartilage, the lung and the vessels[9–11]. Elastin is formed by tropoelastin, a subunit that is synthesised in the cell and then cross-linked with other tropoelastins

to make elastin[12]. Proteoglycans are a group of proteins with several functions, such as several cell-interactive functions and collagen fibril formation[13]. Proteoglycans are composed of a core protein and attached glycosaminoglycan chains. These proteoglycans can be important co-receptors[5,14], can store signalling molecules inside of the ECM to mark specific regions[15] or can be released during an injury[16]. The final major component of the ECM is the laminins, which consist of three chains, alfa, beta and gamma, creating a large, over 400 kDa protein[17]. Laminins have a multitude of functions such as cell attachment, growth, differentiation, and organisation of the ECM, therefore, the deletion of laminins is almost always lethal[18,19].

Distal lung tissue - its extracellular matrix and alveolar cells

The distal part of the lung tissue consists of the basement membrane, a thin ECM comprising elastin, laminins, collagens and proteoglycans[20]. It is an interconnected structure with pores to facilitate gas exchange, where oxygen is taken up and carbon dioxide is released. A layer of alveolar cells covers the extracellular matrix. There are two types of alveolar epithelial cells, alveolar type 1 and alveolar type 2. Even though alveolar type 1 constitutes only 8% of the total number of cells in the lung, they cover over 95% of the area. Its main function is to create a tight junction epithelial layer, which is thin enough to facilitate gas exchange but strong enough to keep the outside bacteria, allergens and dust at bay[21]. Alveolar type 2 cells have two functions, to secrete the surfactant proteins B and C that function to reduce the surface tension to facilitate gas exchange and to avoid the alveolus from collapse[22], and to proliferate and differentiate into alveolar type 1 to replace lost cells. The alveolar type 1 cells are subspecialised, losing their ability to divide.

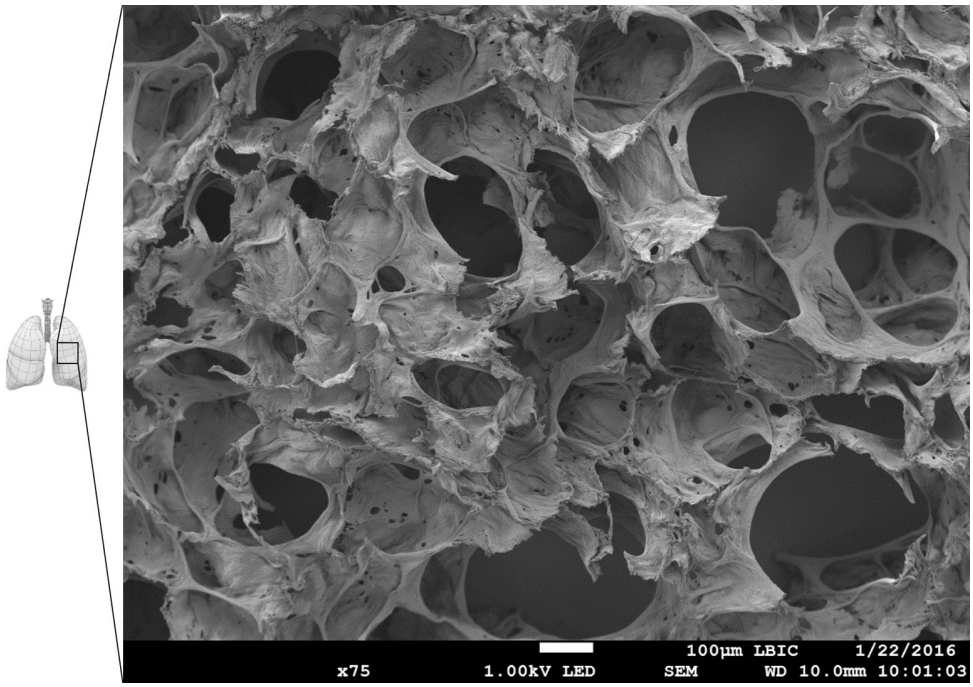

Figure 1 Decellularised distal lung tissue showing the porosity and interconnectivity of the extracellular matrix in the lung. The photo was taken by Linda Elowsson

Glycosaminoglycans

Despite being molecules that are hard to study, glycosaminoglycans play vital roles in the ECM. Glycosaminoglycans are long linear polysaccharides of repeating disaccharide units with various functions. They are an important part of the cell surface, forming together with other carbohydrates the glycocalyx, which, for example, protects us from viral infections[23,24]. Due to their negative charge, the glycosaminoglycans bind and retain a considerable amount of water, making them perfect for shock absorption by contributing to the viscoelastic properties, being found to a large extent in cartilage[25]. Another important role of the glycosaminoglycans is that they act as traffic lights in the body, through the binding and releasing of signalling molecules, such as growth factors, in the extracellular matrix, creating gradients to steer cells[26]. Additionally, to create gradients of growth factors, glycosaminoglycans act as co-receptor for several growth factor receptors[27].

Growth factors

Understanding that glycosaminoglycans have a multitude of properties, this thesis has focused on their interactions with growth factors. Growth factors are signalling molecules used if there is a need to send signals over large distances in the body, to create a gradient to guide cells to a specific site or away from a specific location[28],

or to store a signal at a specific place to be released in case of an injury[29]. There are numerous growth factors in the body, and each one would need its thesis to understand all aspects of their functions, but below is a brief introduction to the ones covered in this thesis.

Hepatocyte growth factor (HGF), also known as scatter factor, was first cloned in 1989[30]. One of the most interesting functions related to this thesis is its proliferative and migrating effect on epithelial cells and release after injury in the repair process[31–33].

For the successful regeneration of most tissues, there is a need for the formation of new blood vessels. Fibroblast growth factor 2 (FGF2)[34], vascular endothelial growth factor (VEGF)[35] and platelet-derived growth factor (PDGF)[36] are three of the main factors in neo-angiogenesis, where FGF2 and VEGF are primarily responsible for inducing the new formation of blood vessels, and PDGF induces the maturation of newly produced blood vessels so that the blood can flow through[37].

The family of bone morphogenetic proteins (BMP) has, despite its name, a multitude of essential functions in other organs such as the lung. During development, BMP4 is the key guiding molecule during lung budding formation[38]. Additionally, BMP4 is important in the adult lung. Evidence in mice suggests that BMP4 is the driver of alveolar type 2 cells changing from a proliferative to a differentiative state and becoming alveolar type 1 cells[39].

Mesenchymal stromal cells

There is a continuous turnover of the ECM and, thus, a need to continuously produce more. One of the main extracellular matrix-producing cell types is mesenchymal stem cells or mesenchymal stromal cells, which have various functions and plasticity[40]. Mesenchymal stromal cells can differentiate into osteoblasts, chondrocytes and adipocytes[41], and harbour immunomodulatory[42] and regenerative effects in the tissues[43]. These cells were first isolated from blood[44], but recently tissue-specific mesenchymal stromal cells have been studied extensively. One of the reasons is that clinical trials with mesenchymal stromal cells have shown that even though the cells are safe for the patients, little effect on the disease and tissue regeneration has been demonstrated. The cells are taken up in the tissue but are quickly cleared after three days in the body[45]. Thus, more and more attention is being given to the immunoregulatory properties of the mesenchymal stromal cells, through its paracrine actions and possible use in diseases where there is a need for a high but short anti-inflammatory effect, such as for acute respiratory distress syndrome[46]. There is also an increasing interest in the possibility of cell preconditioning to remove or minimise the difference between different donors, and to prime the cells for their anti-inflammatory effects which might get lost during isolation and pre-cultures of the cells[47].

Macrophages

The macrophages have been found to play an important role in tissue remodelling. Macrophages are part of the immune system[48,49] with the main function to phagocyte dead cells or pathogens[50,51]. Depending on the stimuli, these immune cells are also able to polarise to have other functions and are therefore often subdivided according to their functions[52]. M1 macrophages are considered to have an inflammatory phenotype, which upon stimulation of lipopolysaccharides or tumour necrosis factor alfa secretes cytokines such as interleukin-1 beta and interleukin-6[53]. In contrast, M2 macrophages have an anti-inflammatory or regenerative phenotype secreting factors such as PDGF, transforming growth factor-beta, insulin-like growth factor 1 and VEGF[54]. Furthermore, M2 macrophages can be subcategorised as M2a which is characterised by its parasite-killing effects[55], M2b as an immune regulatory phenotype[56], M2c with a focus on tissue repair and matrix remodelling[55] and M2d which is defined as more pro-angiogenic[57].

Paper I: hLMSC Secretome Affects Macrophage Activity Differentially Depending on Lung-Mimetic Environments

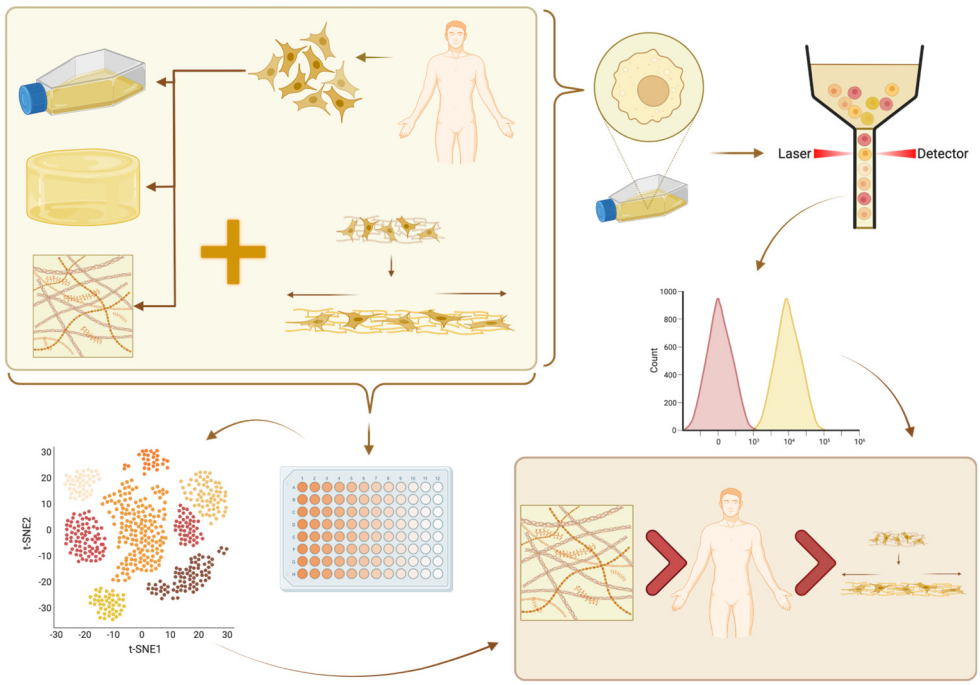

Figure 2 In this paper we used patient-derived mesenchymal stromal cells, which were cultured in three different scaffolds and combined with and without stretch. We had two main readouts, which were a Luminex assay of the supernatant of the mesenchymal stromal cells and analysed using a bioinformatic approach to gain a deeper understanding of how the culture conditions changed the cells. Secondly, we stimulated macrophages with the supernatant of the mesenchymal stromal cells and characterised the cells using flow cytometry. Combining the data from these two techniques, we were able to see that the largest change was due to the type of 3D environment used, followed by the patient heterogeneity of the cells. Finally, the smallest effect came from the addition of stretch. [58]

In paper I, we wanted to gain a better understanding of three aspects. First, the importance of the interplay between the ECM and the cells and the impact of the morphology of the 3D environment. To date, most of the studies focus on using decellularised tissue turned into a hydrogel, but the lung is porous in the naïve state. Therefore, we wanted to evaluate the difference between the effects of an extracellular matrix hydrogel and the porous naïve extracellular matrix have? Secondly, we wanted to observe how mimicking the breathing pattern of the lung in these different environments changed the cellular response. Finally, we wanted to study the impact of the patient variability and whether any of the conditions would push the cells to respond more homogeneously.

The cells were isolated from healthy human lung tissue and cultured in three different microenvironments either under cyclic stretch or under static conditions. The different culturing conditions were: (i) 350 µm thick decellularised porcine lung slices (L-scaffold), (ii) hydrogels made from decellularised porcine lung tissue (L-hydrogel) and (iii) standard 2D polystyrene culture dishes (Plastic). Samples under cyclic stretch were stretched 20% every five seconds to mimic physiological conditions. All combinations of conditions were cultured for five days and a selection of immunoregulatory and regenerative proteins in the culture medium was evaluated using Luminex assay. Using a bioinformatics approach, we could see that the largest difference in the secretome could be observed between different culturing conditions followed by patient difference and lastly the stretch difference. The secretome profile of hydrogel and plastic overlapped to a great extent while the decellularised porcine lung slices showed a more distinct profile. To confirm these findings, we removed the culture medium and stimulated the macrophages to evaluate the induced immunoregulatory effect of the mesenchymal stromal cells in different conditions. We identified the macrophages into M1, M2a, M2b and M2c using flow cytometry, and a general trend of more M2 differentiation was observed when stretch was applied. Additionally, we could see that the M1 population decreased in the decellularised porcine lung slices. Taken together, the data highlighted the importance of knowing how different factors interact, even if there is a large difference in culture conditions.

Secretome analysis using the Luminex assay

It was important to analyse the composition of the protein secretome as it is known that the secreted proteins from mesenchymal stromal cells can influence the immune response. The enzyme-linked immunosorbent assay (ELISA) is a method to detect a single type of molecule using an antibody. Because there is often a need to analyse various proteins, Luminex developed a multiplex platform to detect several proteins at the same time, and has become synonymous with a multiplex ELISA[59] The method is based on a sandwich ELISA, with two antibodies binding to each molecule. One set of these antibodies is bound to magnetic beads that with a magnet are pulled to one side to easily wash away unwanted proteins. Subsequently, these beads have two different dyes and the amount of dye in each bead acts as a barcode. The amount of the first dye gives a number from one to ten. Equally, the amount of the second dye gives a number from one to ten, which gives rise to 100 unique beads. For each bead, there is an additional detection antibody to confirm the binding of the protein. Luminex is a widespread technique due to its relatively low price and the possibility to detect very low amounts of protein in samples. When Luminex was formed in 1995, the detection of light was one of the low cost detection methods, but as other techniques have become cheaper, today, it is possible to multiplex with DNA barcode instead of light barcodes at a low cost, which allows looking at thousands of proteins at the same time[60].

Bioinformatics on Luminex data

The data obtained from a Luminex assay often originates more data points for each biological sample than the number of biological samples, resulting in many of the classical statistical methods failing to be effective[61]. To solve this issue, one can use a technique called dimension reduction, referred to as uniform manifold approximation and projection (UMAP)[62]. Briefly, the UMAP algorithm reduces the number of dimensions by finding points with similar values in the data set, which creates clusters to fit in a 2D plot. We used UMAP to analyse our Luminex values to get a sense of which samples clustered together. The main factor for clustering came from the cell culture environment, followed by the patient variability, and, finally, the comparison between stretched and static samples clustered very close together. By comparing stretch to static, we could observe a reduction in both interleukin-6 and tumour necrosis factor-alpha with the addition of stretch, highlighting that even if this was not a major effect it was consistent.

Investigating the immune properties of the secretome using flow cytometry

To follow up on the Luminex data, we stimulated macrophages with medium from the different culture conditions of the mesenchymal stromal cells and analysed the macrophage differentiation using flow cytometry. This method is based on antibodies that bind to proteins on the surface of the cell, where each cell type, or macrophage subtype in our case, has a unique set of antibodies marked with fluorochromes. The cells are then processed and exposed to laser light. If present on the cell surface, the antibodies are activated and emit a fluorochrome signal resulting in the determination of protein expression levels in each cell. One of the main drawbacks of flow cytometry is that you need to have an antibody and a unique fluorochrome for each protein you want to study, which limits you to a maximum of approximately 40 proteins per cell. Moreover, proteins must be on the cell surface and thus intracellular proteins cannot be studied without the permeabilisation of the cell. In contrast, flow cytometry allows for millions of cells to be quickly analysed and to determine many cell types and states from relatively few proteins presented on the surface, allowing to conclude cell functions from just a few surface proteins.

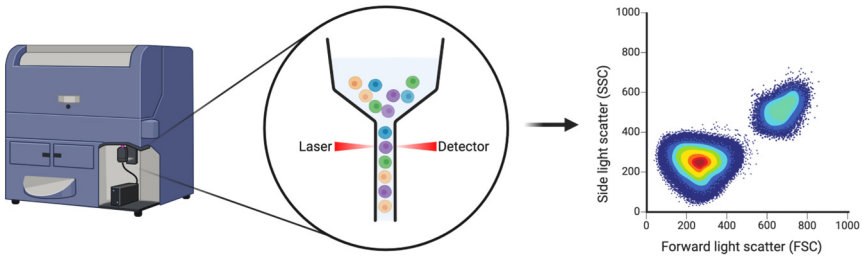

Figure 3 In flow cytometry, a laser shines through each cell and a detector can detect any fluorochrome-linked antibody as well as the granularity and size of cells. With the knowledge of which surface markers a cell express, one can determine its type function.

Significance of the spatial composition of the extracellular matrix

We can conclude from paper I that even if you culture cells in the same extracellular matrix, you obtain distinctly different responses whether the extracellular matrix remains structurally intact or whether it is made into a hydrogel. This is in contrast with a field that is focused on cultures in hydrogels based on decellularised extracellular matrix. As we move into more and more complex systems, I believe that it is important to focus on the spatial aspect of the extracellular matrix microenvironment to give different signals to different cells; for example, to give endothelial signals to endothelial cells, and epithelial signals to epithelial cells, and that it results in alveolar type 1 and type 2 cells being surrounded by blood vessels and not the other way around. This insight armed us with the knowledge to try to create a macroporous scaffold, which mimics the distal part of the lung, functionalised with cell-instructive factors directed to a distinct cell type, as we observe in paper II.

Engineering a modular extracellular matrix

"If your science experiment needs a statistician, you need to design a better experiment."

— Ernest Rutherford.

The main aim of my PhD studies was to engineer a completely defined system to induce tissue regeneration with a focus on distal lung tissue.

There are two ways of engineering a defined system, either you use a top-down or a bottom-up approach. In a top-down approach, you slowly break down the end product into smaller and smaller components. Taking serum as an example, it increases the proliferation of cells, but the composition of factors that contribute to this effect is unknown. One would then fractionate the serum to find out which fraction world still induces the proliferation of cells. Though the drawbacks of this approach are that one might miss the effects of the mixture, limitations in the isolation techniques that might compromise the molecular integrity, issues with purification from animals, as well as batch differences.

The second approach is using a bottom-up approach, where each factor is defined from the start and added step-by-step to construct a completely defined product. This approach overcomes most of the shortcomings of the top-down approach but introduces other drawbacks as the sheer number of possible combinations that arise when considering that molecules have synergetic effects. Coming back to the serum example, if you were to design a completely defined medium to replace serum, there might be 21 different components that are essential for its composition and therefore need to be optimised. If you were to test 10 different concentrations of each component, that would result in sextillion different combinations, which would take every person on the planet to test one combination every second for the rest of their lives and it would still not be enough. Thus, a bottom-up approach depends on two things, having defined the components to eliminate any batch effects and the knowledge of other researchers. In addition, we can be smart about each step until the desired effect is obtained; therefore, not needing to screen every combination.

A combination of the two approaches often results in the best outcome, where one can screen complex mixtures for hits, which can then be added to a controlled and defined system where the exact mechanism can be eluded.

As in paper I and in most of the field, decellularised tissue was used to study the regeneration of tissue. This is a good example of using the top-down approach, which has opened up a new way of culturing cells and resulted in numerous discoveries. What we wanted to do was to utilise this knowledge and take the next step instead of having a bottom-up approach, where each of these discoveries could be added and tested in a controlled manner step-by-step. As an alternative to using an undefined extracellular matrix-based hydrogel system in paper I, we chose an engineered biomaterial as a base that has already been reported to be biocompatible *in vivo*[63]. It is based on a peptide that mimics elastin, an elastin-like recombinamer scaffold, which is tunable both in spatial composition as well as easily functionalised. Pushing this approach further, we screened synthetic glycosaminoglycans for their interaction with growth factors and were able to show that we could differentiate human umbilical vein endothelial cells in a completely defined system, with additional *in vivo* data showing that we were able to instruct cells *in situ*.

Paper II: Controlled Release of Growth Factors Using Synthetic Glycosaminoglycans in a Modular Macroporous Scaffold for Tissue Regeneration

This study aimed to engineer a completely defined modular system for distal lung regeneration. As a base, we started to use a recombinantly produced biomaterial based on human elastin, elastin-like recombinamer (ELR), a hydrogel that is formed through click chemistry and modulated to contain RGD sequences for improved cell attachment and PMGPSGPW, QPQGLAK, and GPQGIWGQ peptides for degradability[64–66]. From this material, we developed a technique to make it macroporous with defined pore sizes, to mimic a wide range of porous organs but with a focus on the lung and alveolar structures[67–69]. We and others have shown the importance of glycosaminoglycans in cell signalling, but to overcome the inherited problem of not knowing the sequence of isolated glycosaminoglycans, we started to evaluate the use of synthetically produced glycosaminoglycans with known sequences[70–72]. Its interactions with growth factors remain unclear due to its novelty. We showed that synthetic glycosaminoglycans with different lengths and sulphation patterns had defined interactions with a range of growth factors and that it could click in the synthetic glycosaminoglycans with the elastin-like recombinamer biomaterial to functionalise the biomaterial with growth factors. In the human umbilical vein endothelial cell differentiation assay, we showed that despite being shorter synthetic glycosaminoglycans, they retained their ability to interact with cell receptors by inducing differentiation. As a proof-of-concept experiment, the

macroporous elastin-like recombinamer scaffold, functionalised with a defined synthetic glycosaminoglycan and immobilised with FGF2, was transplanted in a subcutaneous mouse model. This combination has a slower release of growth factors, and this resulted in a small increase in blood vessel formation and a large change in the immune response showing that it is possible to engineer the desired outcome.

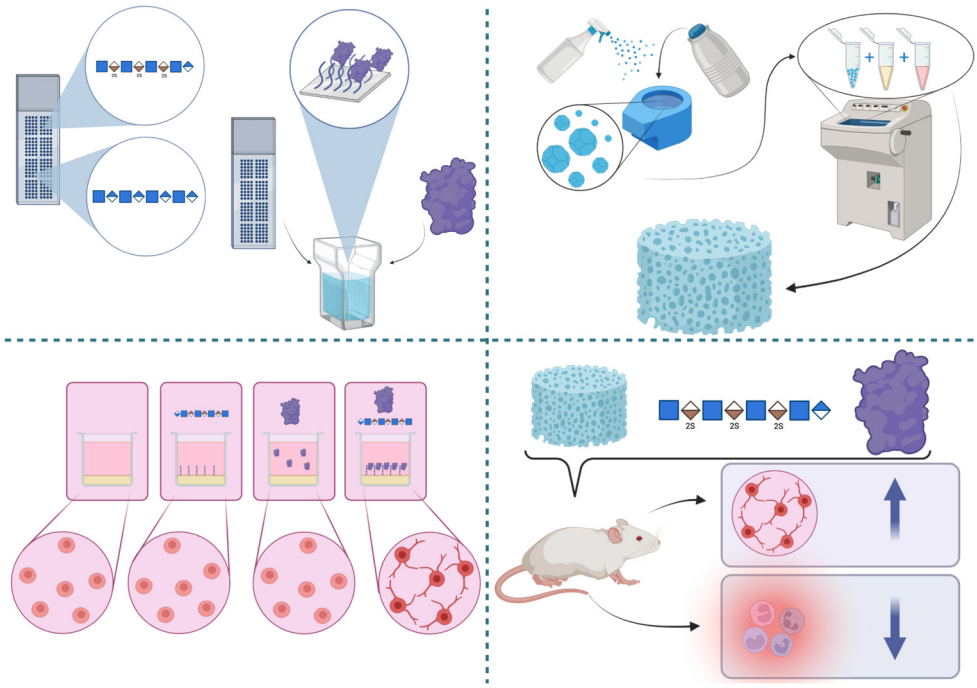

Figure 4 paper II consists of four parts. 1) A range of synthetic glycosaminoglycans were screened for their growth factor interactions using a microarray approach. Six synthetic glycosaminoglycans were selected for further characterisation using surface plasmon resonance to get the complete picture of the kinetics (kon/koff/Kd) between the synthetic glycosaminoglycans and the growth factors. 2) To confirm that the selected synthetic glycosaminoglycans still retained their receptor binding properties, a human umbilical vein endothelial cell differentiation assay was used and demonstrated that the differentiation only occurred in the presence of both the synthetic glycosaminoglycan and the growth factor (FGF2). 3) Parallel to this a macroporous scaffold based on elastin-like recombinamer was developed with the possibility to later click in the synthetic glycosaminoglycans containing an azide tag. 4) The macroporous scaffold was functionalised with the synthetic glycosaminoglycan and functionalised with the growth factor FGF2 and transplanted into a subcutaneous in vivo model. The data demonstrated that the cell-instructive biomaterial seemed to have an increase in vascularisation and a shift in the immune response, towards a more regenerative state, compared to control groups.

Utilising synthetic glycosaminoglycans for a defined system

To date, there are no sequencing methods to characterise glycosaminoglycans, which is one of the reasons why synthetic glycosaminoglycans have been

developed. Using this bottom-up approach, it is possible to know the exact sequence that you are using. Synthetic glycosaminoglycans are made by enzymatically adding one sugar at a time, purifying the product, and then adding the next one[70,71], followed by adding sulfation to the chain. This results in a known sequence of length and sulfation. It also opens the possibility to use a modified first sugar which can either have a fluorochrome, biotin or an azide, thus allowing the use of glycosaminoglycans in the right orientation in downstream applications. Synthetic glycosaminoglycans are proposed to become an alternative to low molecular weight heparin, which is chemically, or enzymatically fractionated heparin purified mainly from pig intestine, to avoid an animal-sourced starting material[73]. It has also opened the possibility to study longer glycosaminoglycans, which has delivered promising drug candidates, for example, against paracetamol overdose and the acute liver failure that follows[72].

Screening of growth factors and synthetic glycosaminoglycans interaction

Microarrays are used for screening interactions of substances, for example, growth factors, with molecules of interest that are bound to specific coordinates on a glass slide. On our slides, 52 different synthetically produced glycosaminoglycans were printed and screened with 9 different growth factors, including VEGF, HGF, BMP4, FGF2 and PDGF-AA. An antibody conjugated with a fluorochrome with specific binding to the growth factor was added to detect which synthetic glycosaminoglycans had bound. Glycosaminoglycans fell into three different groups, showing high binding for all screened growth factors, showing low binding and synthetic glycosaminoglycans which showed high binding to some but not all growth factors. Synthetic glycosaminoglycans from each group were chosen to be further characterised with surface plasmon resonance, which is a lower throughput system but allows us to measure the association and dissociation speed between the growth factors and the synthetic glycosaminoglycans, which allowed us to measure kinetics for 33 growth factors.

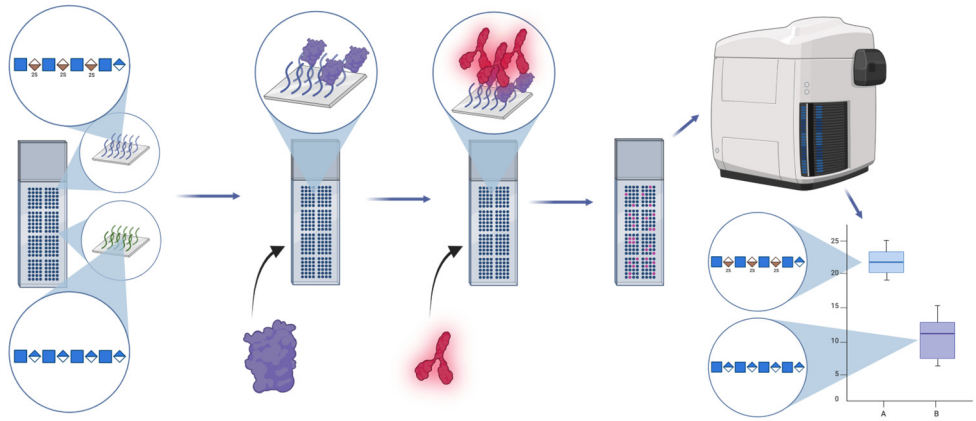

Figure 5 Synthetic glycosaminoglycans were screened using microarray. Each synthetic glycosaminoglycan is spotted at a different location. Firstly, a protein of interest was added, which binds to the synthetic glycosaminoglycans. Secondly, a fluorescent antibody that binds to the protein was added. Finally, a fluorescent scanner was used to find which spots have a high signal of fluorescent antibody, indicating that these glycosaminoglycans bind to the proteins the strongest.

Confirming the biological function of synthetic glycosaminoglycans using human umbilical vein endothelial cell differentiation assay

Human umbilical vein endothelial cell differentiation assay or tube formation assay, as it is often referred to, is an *in vitro* evaluation to study the formation of new vessels. It is an easy and low-cost method that does not capture the complexity of the full formation and maturation of blood vessels but allows the study of single factors as well as conditioned medium. Briefly, the cells are seeded on top of a hydrogel and followed for over 24 h. If the cells differentiate, they start to stretch and organise into tube-like structures where they otherwise stay round and do not move. We wanted to determine whether we could click in the synthetic glycosaminoglycans into the elastin-like recombinamer, and also show that these synthetic glycosaminoglycans, which are shorter than the native ones, still can act as a co-receptor for differentiation and not only to show binding to growth factors. For vascularisation, glycosaminoglycans act as co-receptor to fibroblast growth factor receptors[74]. We demonstrated that both the synthetic glycosaminoglycans incorporated in the elastin-like recombinamer hydrogel and FGF2 were required to obtain the differentiation of the cells, as only one of the factors did not induce the effect, thus highlighting the need for both molecules for the formation of blood vessels.

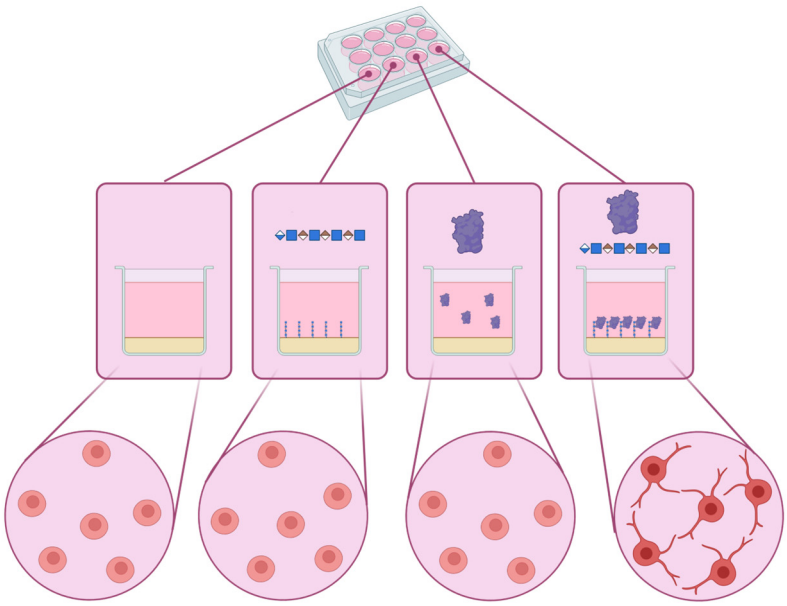

Figure 6 Human umbilical vein endothelial cell differentiation assay consists of placing endothelial cells on top of a hydrogel and waiting for 24 h to see if they form a tube-like structure. What we could see in our experiments was that if we only put synthetic glycosaminoglycans or the growth factor FGF2 no differentiation was observed but by adding both the synthetic glycosaminoglycan and the FGF2, differentiation and tube-like structures were observed.

Using elastin-like recombinamer as a defined extracellular matrix

Elastin-like recombinamer is a biomaterial that is based on human elastin and expressed in bacteria. The elastin-like recombinamer consists of repeating elastin motifs, the sequence is VPGXG where X can be any amino acid other than proline[75,76]. A unique property of elastin-like recombinamer is reverse solvability, so instead of being soluble when temperatures are above the transition temperature, it is soluble when the temperature is lower than the transition temperature. This is utilised in two ways, it makes the purification after expression much easier as the temperature is simply increased above the transition temperature, the elastin-like recombinamer precipitates to the bottom, and the supernatant is removed, which is repeated several times to obtain a pure product. In paper II, these properties were used by mixing the elastin-like recombinamer with ice crystals without the ice melting. For cryogel production, the elastin-like recombinamer was dissolved in 90% phosphate-buffered saline and 10% ethanol to allow a temperature of -5°C without the elastin mixture freezing or the ice crystals melting. Elastin-like recombinamer can be used without any modifications but in paper II we used a click-compatible elastin-like recombinamer [65]. This means that we had one part of the

elastin-like recombinamer where the lysines were modified with a cyclooctyne molecule, referred to as the HE5, and the elastin-like recombinamer where the lysins were modified with an azide referred to as HRGD6. Thus, when these two elastin-like recombinamers mix they would form a covalent bond. Additionally, the HRGD6 contains the amino acid sequence RGD, which is a known cell adhesion motif for integrins to bind to, letting the cells bind better to the hydro and cryogel. The HE5 contains cleavable domains to promote the breakdown and integration into the tissue, these are PMGPSGPW for cathepsin K, QPQGLAK for matrix metalloprotease 13 and GPQGIWGQ for matrix metalloprotease 2 and matrix metalloprotease 9.

Table 1 Amino acid sequences and the molecular weights of the two different elastin-like recombinamers, which were used in this thesis. HE5 was later activated with cyclooctyne and HRGD6 was activated with azide, which allows them to mix with molecules and form a covalent bond.

ELR	Abbreviated amino acid sequence	Mw (Da)
HE5	MGSSHHHHHHHHGLVPRGSHMG-KKK-[(VPGVG)$_2$-VPGEG-(VPGVG)$_2$]$_5$-VGGGGG-PMGPSGPW-GGGG-VGGGG-QPQGLAK-GGGGG-VGGGGG-PQGIWGQ-GGGG-[(VPGVG)$_2$-VPGEG-(VPGVG)$_2$]$_5$-VGGGGG-KKK-GGGGG-[(VPGVG)$_2$-VPGEG-(VPGVG)$_2$]$_5$-VGGGGG-PMGPSGPW-GGGG-VGGGG-QPQGLAK-GGGGG-VGGGGG-PQGIWGQ-GGGG-[(VPGVG)$_2$-VPGEG-(VPGVG)$_2$]$_5$-V-KKK	54426
HRGD6	MGSSHHHHHHSSGLVPRGSHMESLLP-([(VPGIG)$_2$-(VPGKG)-(VPGIG)$_2$]$_2$-AVTGRGDSPASS-[(VPGIG)$_2$(VPGKG)(VPGIG)$_2$]$_2$)$_6$-V	60650

Introducing porosity into the elastin-like recombinamer by optimising a cryogelation technique

The distal lung is a network of alveoli, which is made up of pores ranging from 200 to 500 μm to facilitate the oxygen and carbon dioxide exchange[77]. As we observed in paper I, the location and 3D organisation of the extracellular matrix were very important for the response of the cells. Thus, we wanted to create a synthetic extracellular matrix using the elastin-like recombinamer which mimics the distal part of the lung by being macroporous with interconnected pores also ranging from 200 to 500 μm. There is a multitude of methods for creating macroporous structures but one of the most widely utilised is cryogelation, where the scaffold or extracellular matrix forms around ice crystals, which are then removed by thawing or freeze drying, leaving a porous scaffold behind[78]. My first trials when creating

cryogels simply consisted of mixing the HE5 and the HRGD6 elastin-like recombinamer in tubes dipped in -18°C glycerol. Unfortunately, this created directional freezing, as the bottom was cooled first, which created a more sheet-like structure in the scaffold. To circumvent this, premade ice crystals were added to the mixture. Ice was made by spraying water on liquid nitrogen and then straining the ice through filters with 500 and 200 µm in size. We obtained good results with interconnected pores of the right size and shape but were extremely time sensitive as either the ice crystals melted or the elastin-like recombinamer mixture froze before the reaction could happen. To circumvent these problems and to create a more stable production, the elastin-like recombinamer was mixed with 10 % ethanol to lower the freezing temperature and the procedure was carried out in a cryostat kept at -5°C. This led to much higher yields and more uniform cryogels as there was time for thorough mixing. Cryogels were then frozen at -80°C overnight and finally freeze-dried, resulting in a porous interconnected scaffold.

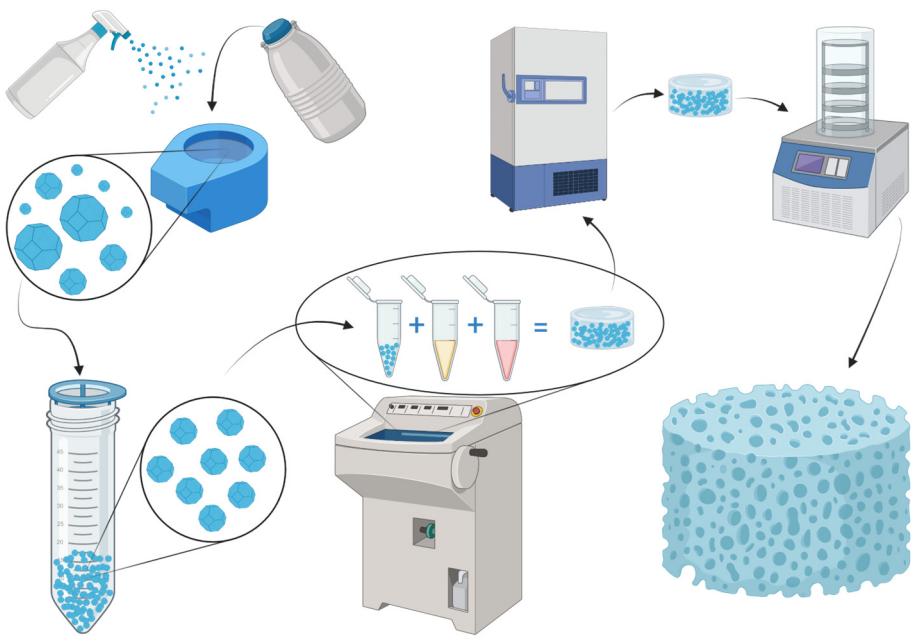

Figure 7 Cryogel production is a multi-step process. First, water is sprayed upon liquid nitrogen to create a heterogeneous mixture of ice crystals. Subsequently, the ice crystals are sieved through strainers which creates a homogenous mixture of the desired size. The ice crystals and the elastin-like recombinamer are placed in a cryostat set at -5°C. These are then mixed and left to completely solidify at -80°C overnight. Finally, the samples are freeze-dried, which removed the ice and left a macroporous structure behind.

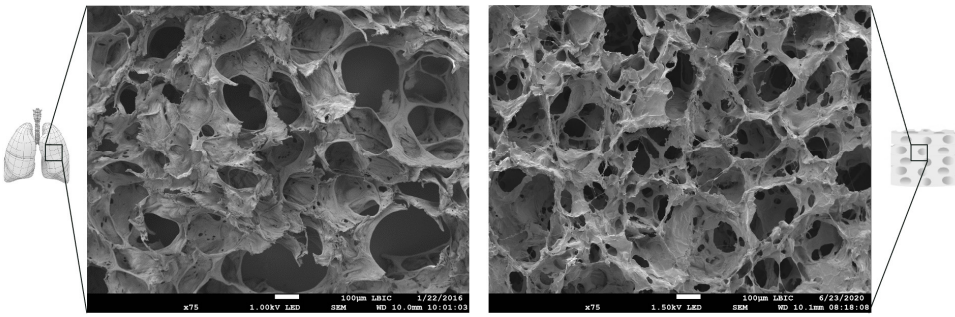

Figure 8 Picture on the left shows a decellularised distal lung tissue. The right picture shows the macroporous elastin-like-recombinamer scaffold. The left picture was taken by Linda Elowsson

Subcutaneous evaluation of the modular macroporous scaffold in vivo

To further characterise the possibility and effect of clicking in synthetic glycosaminoglycans in a macroporous elastin-like recombinamer scaffold we performed subcutaneous implantation in mice. When performing experiments *in vitro* we try to remove all possible parameters that could affect the outcome, focusing on a single core question. Although sometimes there is a need to see and understand how all these factors interplay and what effect it has on the outcome, which is when I believe that we should turn to *in vivo* models. Thus, we evaluated how the slow release of growth factors in a porous cryogel interplayed with the growth of blood vessels and the breakdown of the scaffold over time, resulting in integration or rejection by the immune system. We utilised a relatively simple mice model, a small incision was made on the dorsal side below the thoracic region of the mice, a pocket was created with a blunt pair of scissors, the cryogel was implanted, and the wound was closed with sutures. Samples were extracted after two, four and eight weeks, and plasma was collected.

Evaluation of in vivo data

To evaluate the outcome of the *in vivo* experiment, we utilised two main approaches, we did the same Luminex assay as in paper I, but as a complement to that we used histology. Briefly, histology consists of taking your tissue sample and fixing it in place using paraformaldehyde, a reaction that stops metabolism by cross-linking adjacent lysines. These samples are then embedded in paraffin, both to protect them from oxygen and other environmental factors and also as a physical holder of the samples in the next step. Samples are then sliced on a microtome to produce 3–5 µm thin sections which are put on a microscope slide. Antibodies that bind to the protein of interest are then added to the slides, bind to the protein of interest, and secondary antibodies bind to the primary antibody. The secondary antibody is fluorescent which means that the binding can be visualised in a fluorescence microscope. The addition of FGF2 increased the blood vessel formation, with a

small increase with the addition of glycosaminoglycans. A larger effect was observed when analysing the immune response with the addition of glycosaminoglycans. The combination of both synthetic glycosaminoglycans and FGF2 lead to a higher number of M2 over M1 macrophages indicating a shift in the immune response from a more inflammatory response in the control to a more anti-inflammatory one.

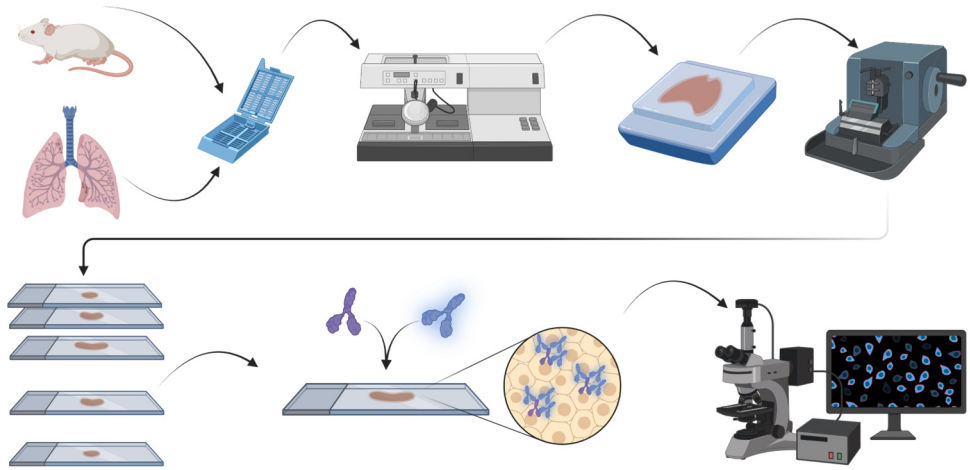

Figure 9 To study thin sections of tissue, histology is utilised. First, the tissue pieces are put into a cassette and are subsequently formalin-fixed and paraffin-embedded. Using a sectioning machine called a microtome, thin sections are produced and mounted on glass slides. To visualise the protein of interest, fluorescent antibodies are added to the slides, which makes the protein of interest fluorescent. This can then be visualised in a fluorescent microscope.

Future scope for synthetic glycosaminoglycans and macroporous elastin-like recombinamer

Paper II demonstrated that synthetic glycosaminoglycans can be used in the field of tissue engineering. They show a large difference in growth factor binding and can thus be finetuned to fit a specific application. They could, for example, deliver HGF a growth factor for epithelial migration into the material, or FGF2, as we have used in our paper, to increase blood formation. We also showed that synthetic glycosaminoglycans have become long enough to interact with cell receptors, to act as co-receptors, demonstrating that synthetic glycosaminoglycans resemble all the functions of native glycosaminoglycan. With the added benefit of knowing the exact sequence that is used and without any need for purification from animals, the regulatory hurdles should be minimal, easing the transition to the clinic when a suitable target is found. One of the main advantages of synthetic glycosaminoglycans is their incorporation of an azide, which ensures the right conformation but also the possibility to be combined with other click-compatible

products, such as elastin-like recombinamer. Having a platform such as the elastin-like recombinamer that can easily incorporate any click molecules of choice, highlights the possibility to fine-tune it to your preference and not just use the provided naïve version. We hope that the ease of use of the elastin-like recombinamer with our developed method for creating a macroporous structure opens up the possibility for the field that studies porous organs to better understand how the structure and spatial composition of the extracellular matrix plays a part in the questions they are trying to answer.

Why is it important to develop new glycosaminoglycan methods?

"What about dermatan sulfate?!?"

-The question Anders Malmström asked at every seminar I went to during my four years

Even though glycosaminoglycan sales are close to 10 billion dollars each year, the glycosaminoglycans research field is relatively small[79]. There are several challenges in the glycosaminoglycan field. Most of the challenges rises from the fact that there is no template for the creation of glycosaminoglycans. Thus, without a template, it has been a challenge to elude the exact mechanism of action for creating the glycosaminoglycans and particularly to understand which stimuli change the glycosaminoglycans. Without a template, the analysis of the sequence has also been limited to either disaccharide analysis using high-performance liquid chromatography (HPLC) or short fragments using mass spectrometry. Finally, it has been difficult studying glycosaminoglycans *in vitro* because the tools have not been compatible with serum, a requirement for culturing most disease cells. In this thesis work, we have tried to tackle the challenge of understanding how glycosaminoglycans are made. Although in my opinion, our biggest contribution to the field is creating a tool, which enables researchers to study glycosaminoglycans *in vitro* in the presence of serum and with a chemical handle that allows picking the desired downstream analysis.

How are glycosaminoglycans made?

There are four classes of glycosaminoglycans; heparan-, chondroitin/dermatan- and keratan sulfate, and hyaluronic acid classified by the sugar units that they contain, with the focus of this thesis project being heparan sulfate[80]. In contrast to RNA and DNA, where the creation of new sequences uses the old one as a template, the creation of heparan sulfate is template-free. It begins in the cytoplasm by activating sugars with uridine diphosphate (UDP), this creates the building blocks UDP-glucuronic acid, UDP-*N*-acetylglucosamine, UDP-xylose, UDP-galactose and UDP-*N*-acetylgalactosamine, which are then transported to the Golgi apparatus. Glycosaminoglycans and thus heparan sulfates are attached to a core protein and

named proteoglycans; furthermore, the glycosaminoglycan chains are linked to the amino acid serine of the core protein. Four sugars are added, xylose-galactose-galactose-glucuronic acid, forming the linker region. This linker region is the same for heparan- and chondroitin/dermatan sulfate. At this point, it is decided which glycosaminoglycan it will become. If an *N*-acetylgalactosamine is added, it becomes a chondroitin sulfate/dermatan sulfate and if an *N*-acetylglucosamine is added, it becomes a heparan sulfate, exactly how this process is decided is not known, but it is known that the amino acids surrounding the serine play a role in this decision[81]. The chain is then elongated with alternating glucuronic acid and *N*-acetylgalactosamine. Finally, chain modifications are made, either glucuronic acid is converted into iduronic acid or a sulfation pattern is added through the donor molecule 3′-phosphoadenosine-5′-phosphosulfate (PAPS), with iduronic acid having the possibility to become sulfated on the second carbon (2S), while *N*-acetylgalactosamine can be sulfated on both the third and sixth carbons (3S and 6S) as well as on the nitrogen (NS)[82].

The GAGome

The exact mechanism for creating glycosaminoglycans inside the cells remains unclear, and one of the reasons for this is that we lack protein structures for most of the enzymes involved in producing the glycosaminoglycans. In an ongoing project, we aimed to characterise the structure of GalNAc4S-6ST, which is involved in the sulfation of glycosaminoglycans and plays a role in fibrosis[83]. Traditionally, to solve a protein structure, the protein needs to form a crystal that arranges the protein in a rigid conformation so that we are looking at one and not multiple conformations. Using a synchrotron, X-ray light is shot at the crystal. The light is then absorbed by the crystal, which forms a detection pattern, and the protein structure can be eluded[84]. Furthermore, glycosaminoglycan enzymes are membrane-bound which is generally known to be harder to crystalise than non-membrane proteins[85,86]. Recently, DeepMind released AlphaFold which is a machine-learning algorithm to turn an amino acid sequence into a 3D structure[87]. It is one of the biggest leaps in protein science in more than a decade, and owing to its capabilities with single protein structure, more focus is now being put on solving protein complexes[88].

Studying glycosaminoglycan enzymes using cryogenic electron microscopy

To overcome the limitations of creating crystals as well as the expensive beam time that is required, new methods are constantly being developed[89]. One such method is cryogenic electron microscopy, in which one takes the protein of interest and put it on a grid, which is then plunge frozen to inhibit evaporation of molecules that interfere with the electron beam, resulting in an ice film less than 100 nm containing the protein of interest[90,91]. Subsequently, millions of photos of the protein are taken using an electron microscope. The reason that several photos were taken is that even when we can zoom in 50000 times, we could not see the proteins. By stacking all

these millions of low-resolution photos of the protein and giving it to the computer, it can calculate a high-resolution protein structure[92].

Determining the structure of GalNAc4S-6ST

A major contribution to glycosaminoglycans' binding properties comes from its sulfation pattern and thus we saw an interest in studying one of these enzymes and we chose GalNAc4S-6ST. Additionally, we hoped that this would gain us the knowledge to later add additional sulfation enzymes to elude the interaction between the enzymes and better understand how different sulfation patterns are created in the body. The function of GalNAc4S-6ST is to add a sulfation group on carbon number 6 when there is already sulfation on carbon number 4 of an *N*-acetyl galactosamine. To study proteins in cryogenic electron microscopy, you need to stack images on top of each other and it is harder to get a protein structure when an enzyme is in different conformations. After a sulfotransferase has transferred a sulfate group from PAPS to its substrate, a by-product called 3′-phosphoadenosine 5′-phosphate (PAP) is formed. If present in excess compared to PAPS, or when PAPS is not present at all, PAP can bind to and inhibit the catalytic function of sulfotransferases such as GalNAc4S-6ST. This can be utilised to make the GalNAc4S-6ST enzyme rigid so that we can capture it with a glycosaminoglycan present at its binding site.

We tried to solve the cryogenic electron microscopy structure for GalNAc4S-6ST with a glycosaminoglycan and PAP present. We were able to get a protein structure that highly resembles the AlphaFold-predicted model (Figure 10 shows the AlphaFold model in blue and our experiment in grey) and had additional densities for the glycosaminoglycan substrate. We were pushing the limits of cryogenic electron microscopy capability by trying to determine the structure of a monomeric asymmetric 50 kDa protein and could not get a high enough resolution to solve the structure.

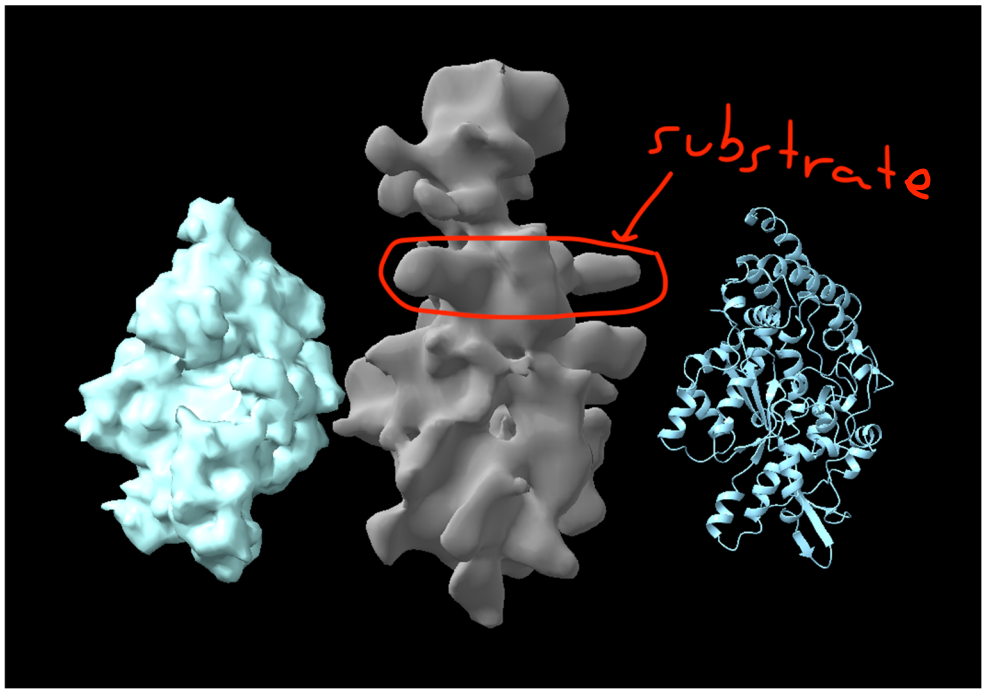

Figure 10 In blue, on both left and right, we can see the predicted alpha fold model of GalNAc4S-6ST. In the middle is our generated volume from cryogenic electron microscopy data and we believe that we can see the substrate bound to the glycosaminoglycans. However, without a higher resolution, it is not possible to be sure. The photo was taken by Emil Weitoft.

Sequencing analysis of glycosaminoglycans

One of the main things holding back glycosaminoglycan research is the lack of tools for sequencing. The most utilised one is disaccharide analysis[93]. This method utilises enzymes, either heparinase or chondroitinase, to degrade the long linear polysaccharides into disaccharide units. When a disaccharide unit is cleaved off, the reducing-end sugar unit reveals an aldehyde in its open-chain form. A fluorophore that selectively reacts with aldehydes, for example, 2-aminoacridone, is added to disaccharides. The mixture is then separated using HPLC with a reversed-phase column and comparing to a mixture of disaccharide standards, thus obtaining the composition of disaccharides in the sample but lacking the order of the original chain or any secondary structures formed.

Mass spectrometry increases the length of the detectable sequence and is routinely used for low molecular weight heparin characterisation but does not allow us to look at complete sequences[94]. By highly purifying proteins of interest, going from 10 kg of tissue to 1 μg of glycosaminoglycans, an average of their glycosaminoglycan sequence has been solved using mass spectrometry, for example, decorin with its length of 40 subunits [95].

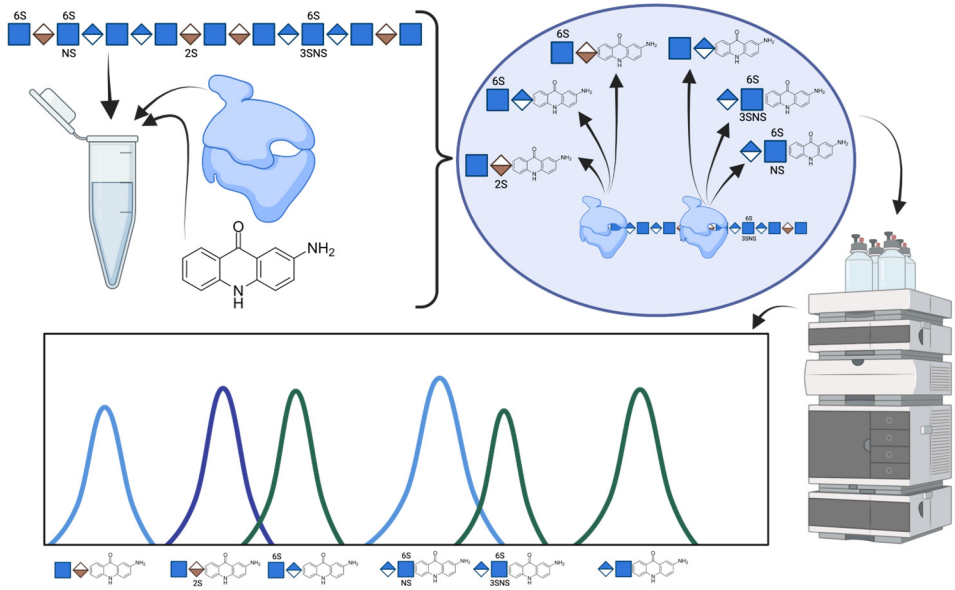

Figure 11 Disaccharide analysis is performed by adding enzymes that break down glycosaminoglycans into disaccharides, disaccharides are then labelled with the fluorochrome 2-aminoacridone and separated in an HPLC machine based on their hydrophobicity.

Paper III: Fluorescently Labelled Xylosides Offer Insight into the Biosynthetic Pathways of Glycosaminoglycans

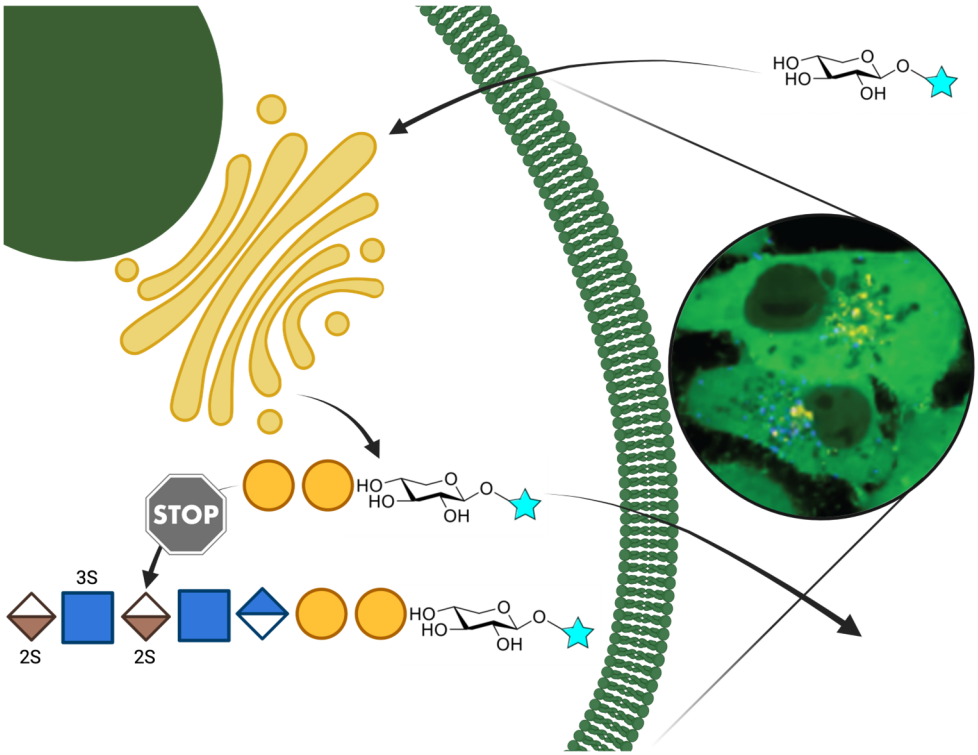

Figure 12 In paper III, we developed a fluorescently labelled xyloside, which we evaluated in part by stimulating cells. The cells took up the xyloside and we could follow this in a confocal microscope. However, the elongation of the glycosaminoglycan chain stopped after two galactoses.

In the third paper, we aimed to develop a tool for glycosaminoglycan research to better understand how glycosaminoglycans move during production and also facilitate downstream application as only newly formed glycosaminoglycans would be fluorescent.

Xylosides were developed in 1973 to better understand glycosaminoglycans. It is a sugar that mimics the initial sugar on the glycosaminoglycans chain, xylose[96]. The xyloside will be taken up by the cell and into the Golgi and the glycosaminoglycan machinery will mistake the xyloside for xylose and will start to build the chain. The xyloside-glycosaminoglycan will then be transported out of the cell and into the medium, which can then be analysed. Generally, more chondroitin sulfate and dermatan sulfate glycosaminoglycans than heparan sulfate are produced using xyloside but this can be modified by chemically changing the xyloside. For example, the heparan sulfate content increases up to 50%[97] by introducing two fused aromatic

rings, creating 2-naphthol-beta-D-xyloside (XylNap)[97]. As these molecules bind to the enzymes that initiate the elongation of the glycosaminoglycan chain, they can be modified to become inhibitors of glycosaminoglycan production[98].

We hypothesised that adding a small fluorochrome on the end of the xyloside would allow us to follow the glycosaminoglycan synthesis in real-time and to study glycosaminoglycans' interaction with other proteins using Förster resonance energy transfer. The selectively fluorescent glycosaminoglycan allows downstream applications such as binding assays. Previously, it has been shown that xylose is the optimal substrate for β4GalT7 and thus chemical modifications to the aglycon have a large effect on the priming efficiency[99]. Several substrates linking the fluorochrome Pacific Blue with the xylose were synthesised with different linker lengths as well as a dithioxyloside. A galactosylation assay was performed on each substrate, which showed an efficient galactosylation up to 0.5 mM and with a significantly higher maximum reaction speed compared to XylNap. Lung epithelial cells, A549, were stimulated with the compounds and analysed using HPLC. No priming of full-length glycosaminoglycans was observed, but it confirmed that galactose was also added to the xyloside in cells. In the cells stimulated with one of the compounds, it was observed, using a confocal microscope, that Pacific Blue co-localised around the Golgi.

There are several possible explanations as to why the Pacific Blue xylosides do not create full-length glycosaminoglycans. It could be because it does not fit in the binding pocket of β3GaT3, which adds the next sugar of the sequence glucuronic acid. As speculated in the article, it could also be due to the charge of Pacific Blue hindering the compounds from entering the Golgi apparatus. A potential use for this work is as an alternative Golgi tracker, but more importantly, it lays the groundwork for further development of new xylosides.

Paper IV: Azide-Functionalised Naphthoxyloside as a Tool for Glycosaminoglycan Investigations

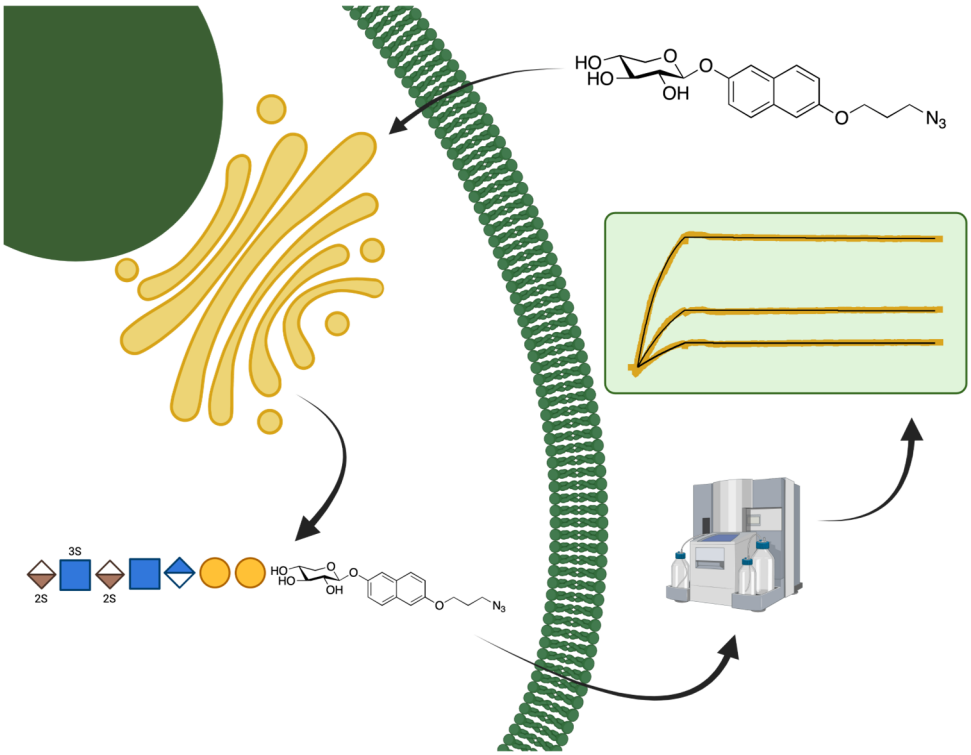

Figure 13 In Paper IV, we coupled a xyloside with a naphthol to increase the ratio of heparan sulfate that is produced as well as an azide. This molecule was taken up by the cell and produced full-length glycosaminoglycans. To show possible uses for this azide-xyloside, we analysed the produced glycosaminoglycans' interactions with hepatocyte growth factor on a surface plasmon resonance and coupled it with a fluorochrome for easier detection.

For the fourth paper (Figure 13), we focused on functionalising the xyloside with an azide to characterise glycosaminoglycans produced by cells using downstream methods, such as surface plasmon resonance. The azide tag is ideal for using click chemistry, a discovery that gave the Nobel prize in 2022, where the azide reacts through a "click reaction" with a ring-strained alkyne such as dibenzo cyclooctyne creating a covalent bound[100]. The reaction is unusual in biology because both azides and alkynes are rare molecules in the body and will not cross-react with anything else. The reaction is fast and works in a wide range of temperatures and pressures, which makes it ideal to use in biology, for example, capturing events in a cell. Additionally, we also utilised 2-naphthol-beta-D-xyloside (XylNap) as a base building block to, in theory, increase the heparan sulfate production in comparison to the chondroitin sulfate and dermatan sulfate [97]. A few different linker sizes

between the XylNap and the azide were considered with the linker size of three being chosen due to possible side reactions for sizes of one and two. The same β4GalT7 assay that was performed in paper III was used again to confirm that the proposed XylNap with azide (XylNapN3) still resulted in galactose being added. When stimulating cells with XylNapN3, the same amount of glycosaminoglycans was produced as with XylNap and a slightly lower amount of sulfation when looking at the disaccharide analysis. By using HPLC, we showed that the glycosaminoglycans produced from XylNapN3 had a more heterogeneous distribution in the chain length.

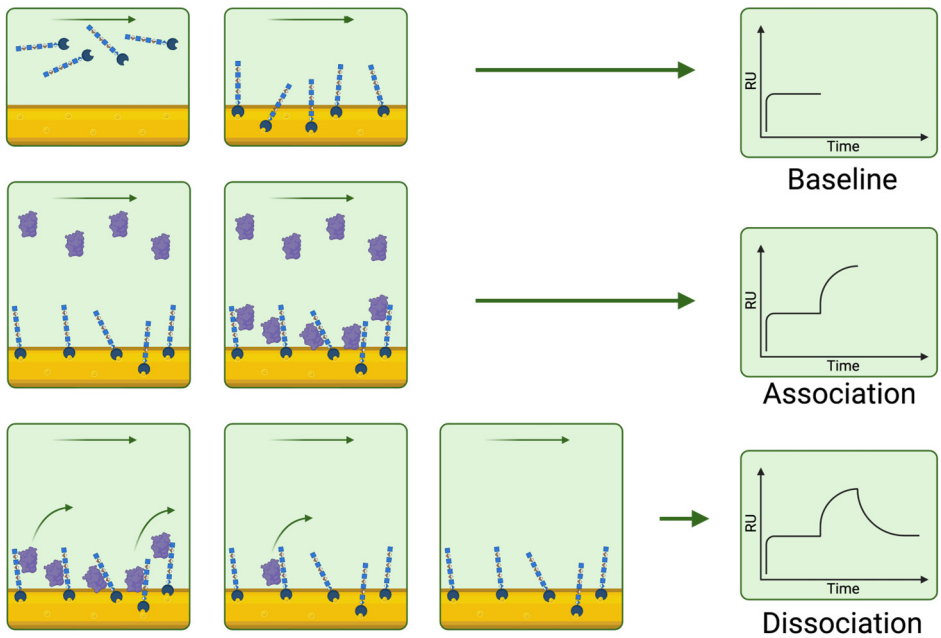

Figure 14 Surface plasmon resonance is performed in three steps. First, a gold surface is functionalised with the molecule that you want to study, named ligand, in our case a glycosaminoglycan. This creates a baseline. Second, a molecule or protein, named analyte, flows over the surface and binds to your ligand, increasing the signal, and giving you the association rate. Finally, only the buffer is flowing over the ligand and the analyte, resulting in the analyte dissociating from the ligand and lowering the signal, giving the dissociation rates.

By having a functional group at the end of the glycosaminoglycans we analysed its interaction with surface plasmon resonance and retained the right conformation of the glycosaminoglycan. Running a surface plasmon resonance experiment consists of three steps, step one is to bind your ligand of choice to the surface, in our case a glycosaminoglycan, which then increases the signal to a new baseline. Step two consists of slowly running your analyte of choice, for Paper IV this was the HGF,

where the signal is increasing as HGF is being bound to the glycosaminoglycans. Step three consists of running pure buffer over the ligand and the analyte and seeing how long it takes for the analyte to fully dissociate from the ligand. Subsequently, the strength of the binding between the analyte and the ligand and the association and dissociation rate constants can be calculated[101,102]. Our results showed that XylNapN3 can be used to gain glycosaminoglycans which can be further analysed with downstream methods, and has a tag on the end for correct binding orientation.

Azide-functionalised naphthoxyloside opens a new possibility for studying glycosaminoglycans *in vitro*, which previously has been lacking. The main advantages are that the glycosaminoglycans produced from the cells can be separated from the glycosaminoglycans already present. This is important because for most cells to grow *in vitro,* there is a need to add serum. The serum is blood that had the cells and clotting factors removed but contains a lot of glycosaminoglycans. The possibility to separate the glycosaminoglycans from the cells and the serum allows us to culture much more cells, especially cells isolated from patients with different diseases. Additionally, studying glycosaminoglycans with an azide tag lets one crosslink it to numerous other methods, such as the elastin-like recombinamer or surface plasmon resonance. Shortly after our publication, Huang et al. published the development of another azide-xyloside but without the 2-naphthol. They showed that amino acids can be exchanged in a protein for unnatural amino acids that have an alkyne tag at the end and thus mix and match glycosaminoglycans and proteins[103]. For glycosaminoglycan research to take the next leap there is a need for a better and more reliable sequencing method and hopefully, the development of nanopore sequencing would solve this[104]. In nanopore sequencing, a single glycosaminoglycan is threaded through a small pore/hole and the theory is that every sugar will give a different resistance when it passes through the pore thus you can measure the resistance and know which sugar molecule it is. This would allow us to know which glycosaminoglycan sequences are more prevalent in disease and thereby get a deeper understanding of how changes in the glycosaminoglycans affect the cells and the surrounding tissue.

To follow cells, harder than it sounds

"Observations often tell you more about the observer than the observed."

- Chris Geiger

Following cells in 2D

During my time as a PhD student, there was a question that was repeatedly asked; how do we track the cells over time and not only look at them at the end of the experiment? For the human umbilical vein endothelial cell differentiation assay, cells were followed for 24 h to see if they formed tube-like structures. Although it is possible to only take a photo at 24 h, automatically taking a photo every hour opens the possibility to more easily troubleshot what is going wrong or to study various biological activities, such as cell proliferation, migration, cell death, or even differentiation. There are several more plug-and-play solutions for following cells in 2D over time. While taking photos in regular intervals is relatively easy, the innovations that are happening now is the use of machine learning to better distinguish cells. Distinguishing individual cells to a high accuracy opens the possibility to follow the cells over time and reliably calculate several factors such as migration, speed and proliferation, and to gain deeper insight by looking at a cell level and not a cell culture level. I think this kind of machine will be standard equipment in almost all cell culturing labs to better understand patient differences as well as clone differences.

Following cells in 3D

Evaluating different dyes

To be able to follow cells in 3D, fluorescent markers are needed, although there are a multitude of fluorescent molecules as well as possible ways to have the fluorescent molecule expressed. When you can stain cells at the endpoint, it is easier to completely stain the cells and dye bleaching over the culture time is not a concern.

One of the first experiments that I did in the lab was to evaluate different cell tracker dyes. These are dyes that are mixed with the cells and then fuse with the cell membrane. During testing two problems became apparent: 1) For longer cultures, the dye was divided in two every time there was a cell division, which after four cell divisions resulted in only a sixteenth of the dye being present in each cell and 2) the dyes were not homogenously distributed in the cells and were showing up as dots in each cell, making it very hard to distinguish cells from noise as well as observing the morphology of cells.

Gene editing with lipofectamine

By making the cells continuously produce a fluorochrome secreted into the cytosol, most, if not all, of the previously mentioned problems would be overcome. There would be no loss of signal over time as the fluorochrome would be continuously produced, and as it is secreted into the cytosol, it would highlight the morphology of the cell. The hard part about this approach is that there is a need for knocking in the fluorochrome gene. To be able to knock in a gene you need three things. Firstly, you need a DNA strand that codes for the gene of interest, for us this is the fluorochrome gene. Secondly, you need a method for the gene to fuse with the genome, otherwise, it will be slowly degraded. This is achieved using clustered regularly interspaced short palindromic repeats (CRISPR) that cut a specific site in the genome and the cell will then try to repair it using homology directed repair. Subsequently, the same sequence will be produced in which case CRISPR will cut again, or it can incorporate our fluorochrome gene in the genome by mistake. Thirdly, a way to get the DNA and CRISPR into the cell is needed. There is a multitude of ways but one of the simplest is the use of lipofectamine, which forms a liposome around the DNA and the CRISPR protein can then fuse with the cell membrane and be incorporated into the cell. However, this method has low effectiveness resulting in the need to select transfected cells after the process. As the cells now produce a fluorochrome we can sort out the positive cells using fluorescence-activated cell sorting to obtain a pure population. Due to the long selection process, this only works on cell lines and cancer cells, as primary cells will move into a senescence state during the selection method.

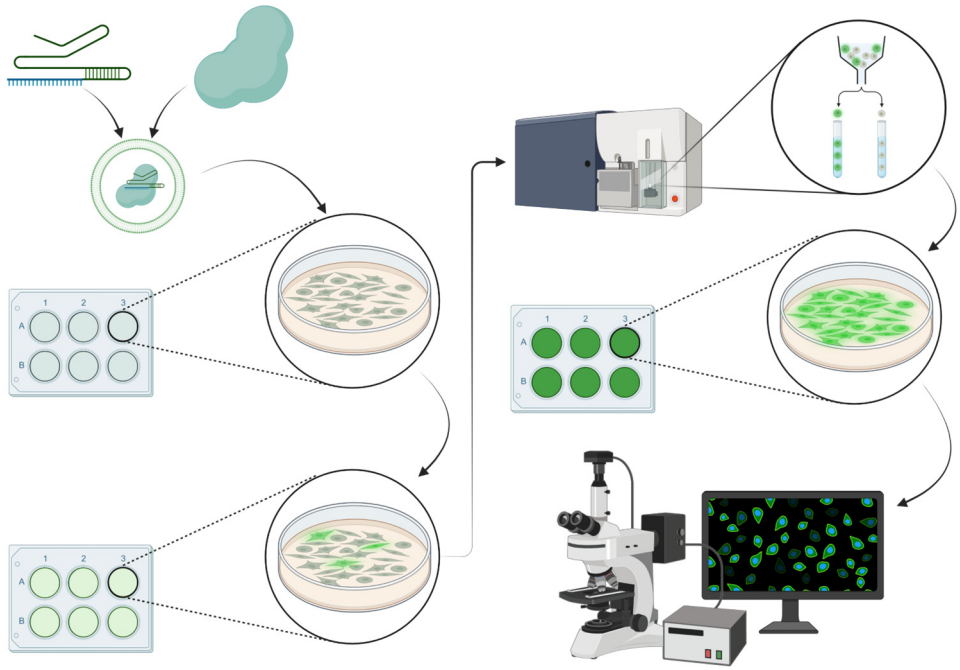

Figure 15 First step of knocking in and out is combining a guideRNA with the CRISPR protein and DNA coding for a fluorescent gene in a liposome using lipofectamine. This is then added to the cell culture which results in some parts of the cells being altered. Then, using flow cytometry, one can use positive selection for knock-in and negative selection for a knockout to create a pure cell culture. The cells can then be used for downstream applications such as microscopy.

The final solution, gene editing with lentivirus

As one of our aims was to follow primary cells in our different types of matrices, we decided to use lentiviruses as the delivery method of CRISPR, which comes with the benefit of having a much higher efficiency during transduction and eliminating the need for selection. Lentiviruses are based on the HIV viruses with almost all proteins removed, but to be on the safe side, the virus is split into three plasmids, and only the cells that have all three plasmids can produce viable lentiviruses, thus eliminating the possibility for lentivirus to spread beyond the first infection. To perform a lentivirus transduction, one starts by producing the CRISPR plasmid that is going to be introduced into the cell by the transfer vector. This plasmid contains the CRISPR gene, the gene for fluorochrome and the guideRNA. The transfer plasmid together with the envelope plasmid and the packaging plasmid are expanded in bacteria. Finally, the plasmids are transfected into a virus-producing cell, often HEK293. The HEK 293 cells will then start to produce functional lentiviruses, which can then be used for transfecting the primary cells to produce fluorescently labelled primary cells.

Utilising the extracellular matrix to culture alveolar cells

"Failure is instructive. The person who really thinks learns quite as much from his failures as from his successes."

- John Dewey

Why is it so hard to culture lung epithelial cells, and why are they important?

Many cell types are hard to maintain in culture. Adding factors to increase proliferation and viability may be effective, but how do you know that the cell which you initially added to the culture will retain its original properties? The culture of distal lung epithelial cells has proven particularly challenging. When removing the alveolar cells from the lung and placing them in a culture flask, the alveolar type 1 cells will not proliferate, thus not making it possible to expand them *in vitro* for further experiments. Placing alveolar type 2 cells in a culture flask will activate its differentiation program into alveolar type 1, instead of its proliferative state.

Which lung epithelial cells can be cultured today?

Already in 1972, there was a need to culture lung epithelial cells and to overcome these challenges that we face today[105]. A crude but effective way to overcome the proliferation problem was to isolate cells from lung cancer tissue, resulting in the cell line A549 and was later repeated in 1982 by isolating pericardial fluid from a cancer patient with papillary adenocarcinoma resulting in the cell line H441[106]. A549 and H441 cells are today widely used due to their ease of culture and fast expansion, facilitating the performance of several experiments in a short time. While these cell lines have helped to gain an increased understanding of the biology of the lung and particularly of lung cancer, they are lacking when it comes to studying regeneration since they are cancer cells. Thus, in 2016, Lehr et al. approached the challenge by infecting primary alveolar type 2 cells with several genes which are known to induce immortalisation and continuous proliferation. The genes which were integrated into the genome were Id2, Id3, E7, Bcl2, Core, Myc, and Nanog, and the immortalised cell line was named hAELVi[107]. hAELVi seems to be more related to the native alveolar cells, as it forms a tight junction monolayer in comparison to the cancer cell lines where the cells grow on top of each other. These

cells became easy to use and retain properties that closely resemble the native state, but as we are still introducing changes in the genome expression there is still room for improvement. During the COVID-19 pandemic, there was a need for relevant lung models and an enormous amount of research and time was spent on developing new ones. Primary mouse alveolar type 2 cells can be put inside of a hydrogel composed of tumour-derived extracellular matrix (organoid culture) together with a stromal cell and they would proliferate and increase in number for over a year in culture[108]. In a very short amount of time, the field was able to go from culturing primary human alveolar type 2 cells in the presence of stromal cells to being able to have a serum-free feeder-free culture of primary human alveolar type 2 cells, which were able to differentiate into primary alveolar type 1 cells upon stimulation with human serum[109]. Although further studies are needed to compare these cultures to their native counterparts and to determine both the molecules and mechanisms by which alveolar type 2 cells differentiate into type 1, which is suggested to be BMP4-dependent in mice but not shown in humans[39]. Culturing primary alveolar type 1 and 2 cells will certainly yield important discoveries, but the lack of available human lung tissue will limit its availability. Thus, considerable research has focused on producing alveolar type 2 cells from induced pluripotent stem cells (iPSC), and while this is a stepwise process that takes time, it would open up the possibility of not using human lungs and to be able to have a greater number of cells available for experiments[110,111]. In 2022, a group was able to push these alveolar type 2 cells to differentiate into alveolar type 1 cells, once again with human serum[112]. I believe it is only a matter of time until every lab can culture iPSCs differentiated into alveolar type 1 and 2 cells to answer their specific question.

Future perspective: Culturing lung epithelial cells in a defined and modular system

One of the major disadvantages of the current methods of culturing alveolar epithelial cells is the commonly used tumour-derived extracellular matrix hydrogel Matrigel, to form organoids. The cells are cultured inside of Matrigel to overcome the fibrosis signalling that the stiff plastic conditions induce, but also lets the cells form a 3D structure. As we demonstrated in paper I, the cell response differed greatly depending on the spatial composition of the extracellular matrix. Matrigel is a brand name for the extracellular matrix solution secreted by Engelbreth-Holm-Swarm mouse sarcoma cells. Thus, it will always be a question about interspecies responses and the fact that it is a cancer cell that produces the extracellular matrix. Perhaps, we are only promoting a proliferative state of the cells in the 3D environment and pushing the cells too far away from their native state. Though the biggest drawback is that Matrigel is a complex mixture, like serum, with an unknown composition that can vary between batches, thus with the risk of introducing new unknown inhibitors and activators. We aimed to optimise our macroporous elastin-like recombinamer scaffold with factors that promote alveolar regeneration. We have done some preliminary cultures and figure 16 demonstrates how hAELVi cells are cultured in the macroporous elastin-like recombinamer scaffold. Firstly, this approach allowed us to evaluate the impact of a macroporous structure on the hAELVi cells. Secondly, it allowed us to easily click in molecules of interest and evaluate the spatial component. Finally, the scaffold has mechanical properties which match the human lung. All these properties allow us to use a bottom-up approach to add one factor at a time and thus gain a deeper understanding of the effect of each molecule and how it interacts with the rest of the system without the need to worry about the effects of unknown components in the hydrogel.

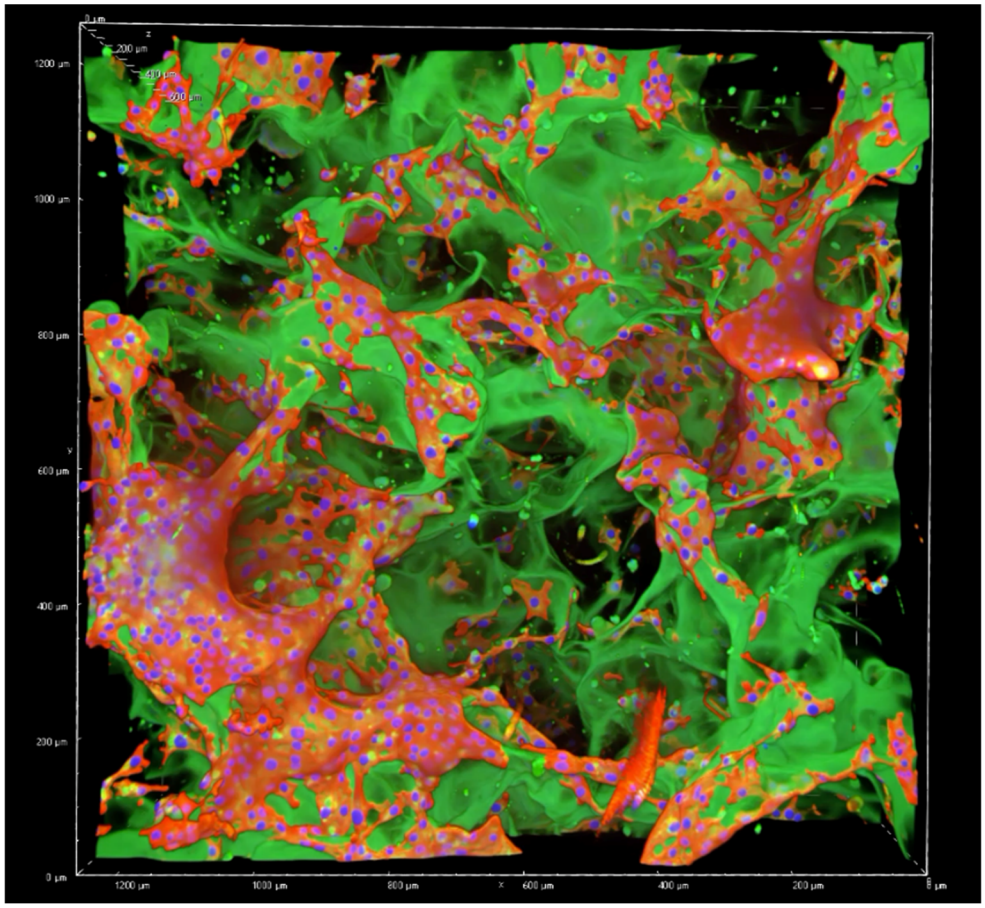

Figure 16 Lung epithelial cell line hAELVi is in red with nucleus in blue cultured on top of a macroporous cryogel made from elastin-like recombinamer in green. The cells are slowly moving inwards towards the centre, creating an epithelial monolayer that mimics the regenerative process in vivo. The photo was taken by Arturo Ibáñez-Fonseca.

Conclusion

In conclusion, paper I focus on understanding the need to mimic the structural properties of the native extracellular matrix of the lung in cell cultures. We studied the impact of retaining the original conformation of the extracellular matrix, the effect the physiological stretch have, and how much the patient variability influences have a different impact. In papers III and IV, the goal was to develop new tools for studying glycosaminoglycans and get a better knowledge of their properties in the extracellular matrix. In paper III, we developed a way to tag the newly produced glycosaminoglycans with a fluorophore. In Paper IV we refined this method to tag the glycosaminoglycans with a clickable tag opening up the possibility to extract newly produced glycosaminoglycans *in vitro*. The method can also be used to investigate how glycosaminoglycans produced by different cell types differ. This thesis has primarily focused on paper II, where all the knowledge from the other papers has been incorporated to create a defined extracellular matrix that can be engineered according to cell attachment, degradation, stiffness and porosity, and can be functionalised with molecules of interest using click chemistry. Using synthetic glycosaminoglycans in our defined extracellular matrix allowed us to get a better understanding of the release kinetics and gradient creation of growth factors. The engineered extracellular matrix opens the possibility to model diseases and orchestrate regeneration.

Populärvetenskaplig sammanfattning

Kroniskt obstruktiv lungsjukdom (KOL) är en sjukdom som drabbar mellan 400 och 700 tusen svenskar och förväntas bli den tredje vanligaste dödsorsaken i världen år 2030. KOL är en sjukdom som framförallt drabbar människor som kontinuerligt utsätts för sotföroreningar, oftast i form av rökning, avgaser från bilar eller fabriker. Människan har en överkapacitet i lungan, alltså att vi får in mycket mer syre per andetag än vi egentligen behöver. På grund av detta så märker man inte av när ens lungkapacitet minskar med tiden. När man väl söker vård för problem med andningen har sjukdomen ofta redan varit aktiv i många år. Det finns ett antal läkemedel som lindrar symptomen vid KOL, såsom luftvägsvidgande, men det finns idag inga läkemedel som hindrar fortskridningen av KOL och inte heller några läkemedel som låter en återfå förlorad lungvävnad.

Det som händer i lungan när man har KOL är att den långsamt bryts ner och man får allt större hålrum. Det är lite som att man skulle börja ta bort delar av en byggställning på en byggarbetsplats. I början påverkas inte arbetet så mycket, men efter ett tag så finns det mindre plats för arbetare vilket gör att bygget går långsammare. Samma sak händer i KOL när byggställningen, vilket i kroppen heter den extracellulära matrisen, bryts ner så att det finns allt mindre plats för cellerna att byta ut koldioxid mot syre, vilket gör att det är svårt att andas.

Den här avhandlingen har fokuserat på lungans byggställning, den extracellulära matrisen. Det slutgiltiga målet är att skapa en byggställning i labbet som sedan kan injiceras i hålrummen i lungan som ett temporärt stöd i syfte att starta igång återbyggnaden av lungan.

I artikel nummer I fokuserade vi på att förstå hur den extracellulära matrisen påverkar celler, vilka faktorer är det som ger störst påverkan. Vi testade att odla cellerna i ett system som härmar lungans rörelse vid andning. Hur skiljer det sig mellan olika individer, hur kommer det sig att vissa drabbas värre än andra och slutligen jämförde vi hur skillnader i formen av byggställningen påverkar cellerna även om de är byggda av samma molekyler.

Artikel nummer III och IV fokuserar på socker, vilket kan kännas lite konstigt när vi pratar om lungan. Men det är så att socker, och specifikt glykosaminoglykaner, fungerar lite som trafikljus i kroppen. Dessa glykosaminoglykaner finns överallt i kroppen i olika former och precis som trafikljus säger de till celler vad de ska göra och vilken väg de ska ta. Glykosaminoglykanerna ger olika signaler genom att fånga

upp olika typer av signalmolekyler och på så sätt kan kroppen ge många signaler samtidigt, men bara ha en typ av socker. Dock är det tekniskt väldigt svårt att studera dessa socker, för vi vet egentligen inte riktigt hur de skapas i cellen. Så i artikel III och IV fokuserade vi på att utveckla nya metoder för att studera dessa socker, glykosaminoglykaner. Med vår metod kan man lättare analysera glykosaminoglykaner som celler producerar genom att vi lagt till ett litet handtag i ena änden där man kan koppla på vad man behöver för analysen, till exempelvis en färgmolekyl, och kan då se var glykosaminoglykaner är någonstans i cellen.

Den sista artikeln, artikel nummer II handlar om att man kan kombinera det vi lärt oss från de andra artiklarna för att ta ett steg på vägen mot ett botemedel för KOL. Här skapar vi en byggställning som efterliknar den byggställningen som finns i lungan, den är ihålig, elastisk och porös så syrgas kan ta sig in. I artikeln skapar vi ett material som kan tryckas ihop för att sedan kunna expandera igen, exempelvis när det trycks ihop genom en spruta. Vi kombinerade detta med glykosaminoglykaner för att ge rätt sorts signaler till cellen att börja bygga upp en ny naturlig byggställning, extracellulär matris. Som ett bevis på att detta fungerar lyckades vi skapa blodkärl i materialet när vi ger trafikljussignalerna för blodkärl, först på labbänken och visade därefter att detta också stämmer när vi testar det i möss.

Vägen kvar till ett faktiskt botemedel för KOL är lång, vi behöver hitta fler av trafikljussignalerna för att samordna de olika typerna av celler som behövs för att få lungan att fungera. Det behöver testas i djur och se att de återskapar rätt typ av lungvävnad och inte bara blodkärl. Dock är detta en viktig del på vägen för att komma dit en dag.

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Figure 17 The fantastic Lung biology group

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